REMARKS / ARGUMENTS

Upon entry of this amendment, the claims pending are claims 1-17, 24-26, and 28-43. Claims 18-23 and 27 were cancelled by previous amendments. Paragraphs (a) (ii) and (b) (ii) of Claims 1 and 43 have been cancelled by amendment. The newly cancelled subject matter from claims 1 and 43 stands canceled without prejudice to refiling as non-elected subject matter, as does the subject matter of Claims 18-23.

Claims 2, 4-9, 11, 28, 30-35, and 37 are amended to clarify the invention in view of the above restriction of the subject matter of Claims 1 and 43. Claims 12 and 38 are amended to incorporate the features of the claims from which they depended, as suggested by the examiner. Claims 16 and 42 are amended to correct minor clerical errors. No new matter was added by these amendments, which are supported in the original specification and by the original claims.

Any subject matter canceled from the claims by amendment is reserved for refiling in a continuation application filed during the pendency of this application. Applicants further affirm the correctness of the inventive entity in view of the cancellation of the non-elected claims.

The specification was amended to insert the sequence listing of wild-type cholera holotoxin subunit A as described below and refer to the same on page 38. The same sequence appears in Mekalanos *et al*, **1983** *Nature*, 306:551-557 in the context of the entire cholera toxin sequence with subunit B and 5' and 3' untranslated regions. The mature subunit A is indicated by the first amino acid appearing under the first mature amino acid "N" in the sequence. SEQ ID NO: 1 is a duplicate of the mature subunit A sequence as set forth in International Patent Publication No. WO 93/13202 (Domenighini), incorporated by reference in the specification at page 38, lines 27-28. Because the sequence of wild-type subunit A was properly incorporated by reference by reference to Domenighini in the specification, no new matter is added by these amendments.

Claim Rejections based on 35 USC §112, first paragraph

> Claims 12 and 38 are rejected as allegedly failing to comply with the enablement requirement and containing subject matter not described in the specification in such a way as to show possession of the claimed invention. Specifically, the examiner states that the plasmid DNA encoding HSV gD2 antigen has not been described. The examiner requires the applicant to amend the disclosure to include the material incorporated by reference. The amendment must be accompanied by an affidavit or declaration executed by the applicant stating that the amendatory material consists of the same material incorporated by reference in the referencing application.

Applicants respectfully request reconsideration and withdrawal of these grounds for rejection in view of the above amendments to the claims and the following remarks.

Claims 12 and 28 do not require the use of ***any specific*** plasmid, merely ***any*** plasmid encoding HSV gD2. It is not the nature of the *plasmid* itself that makes the claims inventive, but rather the ***combination*** of (a) a plasmid encoding the HSV gD2 antigen, with (b) a polynucleotide sequence encoding the ***specifically claimed*** mutant cholera holotoxin.

The identity of the plasmid backbone is *irrelevant* to this claim and Applicants are puzzled by the examiner's insistence upon a claim to a specific plasmid. Plasmid backbones for insertion of antigens, such as HSV gD2, have been well known for years prior to the priority date of this application. The sequence of HSV gD2 has been similarly known and published prior to this priority date.

As previously stated, even plasmids containing sequences encoding HSV gD2 were available in the prior art as of the filing date of this application. The citation to Pachuk, C. *et al*, **1998** *Curr. Topics Microbiol. Immunol.*, 226:79-90 was referenced in this application merely to cite an example of a suitable plasmid. Still other exemplary HSV gD2 plasmids were described by others in the prior art before the priority filing date of this application. See e.g., Nicola *et al.*, **2996** *J. Virol., 70*:3518-3822.

The recitation of a specific HSV gD2 encoding plasmid is not essential to the practice of this invention, where one of skill in the art may select from among available and publicly described plasmids. What is new and non-obvious in this

present application is ***the combination*** of a plasmid expressing HSV gD2 ***and*** a polynucleotide expressing the claimed mutant cholera holotoxin. That this is true is evidenced by the lack of prior art cited against the ***combination***s of elements in the claims.

Applicants respectfully request that the examiner reconsider the grounds for this rejection. Thus, this rejection may be properly withdrawn.

Claim Rejections based on 35 USC §112, second paragraph

> Claims 1-17 and 24-43 are rejected under 35 USC §112, second paragraph, for alleged indefiniteness in view of lack of a reference sequence for the position numbers in the claims.
>
> The examiner asserts that the cholera toxin subunit A is known to have sequence variation, referred to Swiss Prot Accession Nos. Q8vL16, Q81356, and P01555, and thus a reference sequence is necessary.
>
> The examiner states that Applicant is required to amend the disclosure to include the material incorporated by reference, including a declaration as stated previously.

Applicants respectfully request reconsideration and withdrawal of these grounds for rejection in view of the above amendments to the claims and the following remarks.

As stated in detail below, the sequence of CT-A, both wild-type (WT) and variants, are known in the art. All variants of CT-A contain a glutamic acid at position 29. Further, position 29 is clearly identified in the specification by reference to its native glutamic acid residue. One cannot locate a position 29 having a glutamic acid unless one counts from the native protein amino acid 1, not from the well-known 18 amino acid signal sequence of CT-A

Regarding the identity of the amino acid position numbers, Applicants draw the examiner's attention to the documents cited in the specification, i.e., Mekalanos *et al*, **1983** *Nature*, 306:551-557, (as "Bibliography entry 1" on page 2, lines 3-4 and as the first citation on page 114, labeled "Bibliography") and Domenighini et al., International Patent Publication No. WO 93/13202 (page 38, lines 10 and 27). Mekalanos is the standard reference in the art for the well-known sequence of cholera

toxin and its subunits. Further, Domenighini also provides the amino acid sequence of the same mature CT-A subunit cholera toxin and its subunits. Such sequences are also available in the NCBI database, as submitted by the authors of the above-noted publication.

Thus, in publications throughout the art, a reference to position 29 of wild-type cholera toxin subunit A is understood by those of skill in the art to mean the highlighted amino acid in the well-known sequence of subunit A. Note that in a variety of publications, the authors use the same convention for identifying amino acid positions of cholera toxin subunit A, i.e., by identifying the amino acid by position number with a reference to Mekalanos. Such publications did not feel it necessary to set out the well-known sequence. See, e.g., International Patent Publication Nos. WO 97/02348 and WO 97/29771 and background references cited therein; and Vadheim K.L, *et al*, **1994** *Microb. Pathog., 17*(5):339-46.

The specification of the present application clearly states on page 4, lines 4-6 that the present invention is performed using "...a mutant cholera holotoxin featuring a point mutation at amino acid 29 of the A subunit, wherein the glutamic acid residue is replaced by an amino acid other than aspartic acid". ***Applicants' only requirement*** is that the ***amino acid at position 29*** of the sequence of cholera holotoxin A subunit be ***a glutamic acid***.

While Applicants agree with the Examiner that cholera toxin subunit A is known to have sequence variations (e.g., Swiss Prot Accession No. P01555, Q8VLI6, and Q8L356), the sequences of all of these cholera holotoxin subunit A variants ***contain a glutamic acid at position 29***. Therefore, Applicants' claims properly encompass the substitution of any CT-A with a glutamic acid at position 29.

Applicants respectfully disagree with the Examiner's statement that:

> "[i]t is not clear whether the A-subunit could be in the pro-toxin form or in the form where the signal sequence has been cleaved" and that "...the numbering from the N-terminal of the protein antigen would result in a different position for substitution of the amino acid" at position 29.

One of skill in the art would readily understand, based upon the teachings of Mekalanos in 1983 (see the mature CT-A sequence in Domenighini), that the sequence of cholera holotoxin contains an N-terminal 18 amino acid signal sequence

and that the mature protein begins at the asparagine amino acid position 19 (see page 552, paragraph 1, lines 6-7). Further, based on the teachings of the instant specification that position 29 of the amino acid sequence of cholera holotoxin must be a glutamic acid, the sequence thereby cannot possibly include the N-terminal 18 amino acid signal sequence.

In an effort to place the application in condition for allowance and as suggested by the Examiner, Applicants have amended the specification by inserting the amino acid sequence of the wild-type cholera holotoxin subunit A as recited in Mekalanos and Domenighini, amended certain paragraphs in the specification on page 38 to refer to the sequence of the same as SEQ ID NO: 1, and enclosed a Declaration executed by the below-noted attorney of record asserting that the amendatory information being inserted was properly incorporated by reference.

In view of these amendments and remarks, Applicants submit that all claims are now in condition for allowance and that this rejection may be properly withdrawn.

Claim Objections

> Claims 12 and 38 are objected to because they depend from sections of the independent claim which have been withdrawn from consideration. Amendment in independent form would obviate this rejection.

As suggested by the examiner, Claims 12 and 38 have been amended into independent form by incorporating the features of the claims from which they depend. This objection may be properly withdrawn.

In view of the above claim amendments and remarks, Applicants respectfully submit that the claim rejections have been overcome and that the present application is in condition for allowance. Accordingly, allowance of the present application is respectfully requested.

The Director is hereby authorized to charge any deficiency in any fees due with the filing of this paper or credit any overpayment in any fees to our Deposit Account Number 08-3040.

Respectfully submitted,

HOWSON AND HOWSON
Attorneys for Applicants

By Mary E. Bak

Mary E. Bak
Registration No. 31,215
Spring House Corporate Center
Box 457
Spring House, PA 19477
(215) 540-9200

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.	: 09/806,370	Confirmation No. 8568
Applicant	: Randall K. Holmes et al	
Filed	: October 3, 2001	
TC/A.U.	: 1645	
Examiner	: Virginia A. Portner	
Docket No.	: 33,383-00	
Customer No.	: 38199	

Mail Stop AF
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

June 8, 2004

DECLARATION

Sir:

I, Mary E. Bak, residing at 1415 Comly Court, Maple Glen, PA, 19002, a citizen of the United States of America, do declare and state that:

1. I am one of the named attorneys of record in the above-identified patent application

2. This Declaration is submitted in the above-identified application in response to the Examiner's rejection under 35 USC § 112, first paragraph in the Office Action dated March 10, 2004

Express Mail No. EU531570125US

3. Specifically, this Declaration is submitted to support the insertion by amendment of SEQ ID NO: 1 into the specification, which is the mature wild-type cholera holotoxin subunit A sequence as set forth in Domenighini *et al.*, International Patent Publication No. WO 93/13202 (hereinafter Domenighini) cited in the specification at page 38, line 10 and properly incorporated by reference. See, Exhibit A attached herewith.

4. The same sequence appears in Mekalanos *et al.*, **1983** *Nature*, 306:551-557 (hereinafter Mekalanos) cited in the specification at page 2, line 4 in the context of the entire CT sequence with subunit B and 5' and 3' untranslated regions. See, Exhibit B. The mature subunit A is indicated in Mekalanos by the first amino acid appearing under the first mature amino acid "N" in the sequence. SEQ ID NO: 1 is a duplicate of the mature subunit A sequence as set forth in both Domenighini and Mekalanos.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: June 8, 2004 By: Mary E. Bak

Mary E. Bak

EXHIBIT A

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/00, 15/31, A61K 39/106
A61K 39/108, C12P 21/02
C12N 1/21 // (C12N 1/21
C12R 1:19)

A1

(11) International Publication Number: WO 93/13202

(43) International Publication Date: 8 July 1993 (08.07.93)

(21) International Application Number: PCT/EP92/03016

(22) International Filing Date: 30 December 1992 (30.12.92)

(30) Priority data:
MI91A03513 31 December 1991 (31.12.91) IT

(71) Applicant *(for all designated States except US)*: BIOCINE SCLAVO SPA [IT/IT]; Via Fiorentina, 1, I-53100 Siena (IT).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : DOMENIGHINI, Mario [IT/IT]; Via Ungaretti, 17, I-53010 Quercegrossa (IT). RAPPUOLI, Rino [IT/IT]; Via Calamandrei, 39, I-53010 Quercegrossa (IT). PIZZA, Mariagrazia [IT/IT]; Via Colombini, 30, I-43100 Siena (IT). HOL, Wim [NL/US]; 18332 57th Avenue, N.E., Seattle, WA 98155 (US).

(74) Agent: HALLYBONE, Huw, George; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

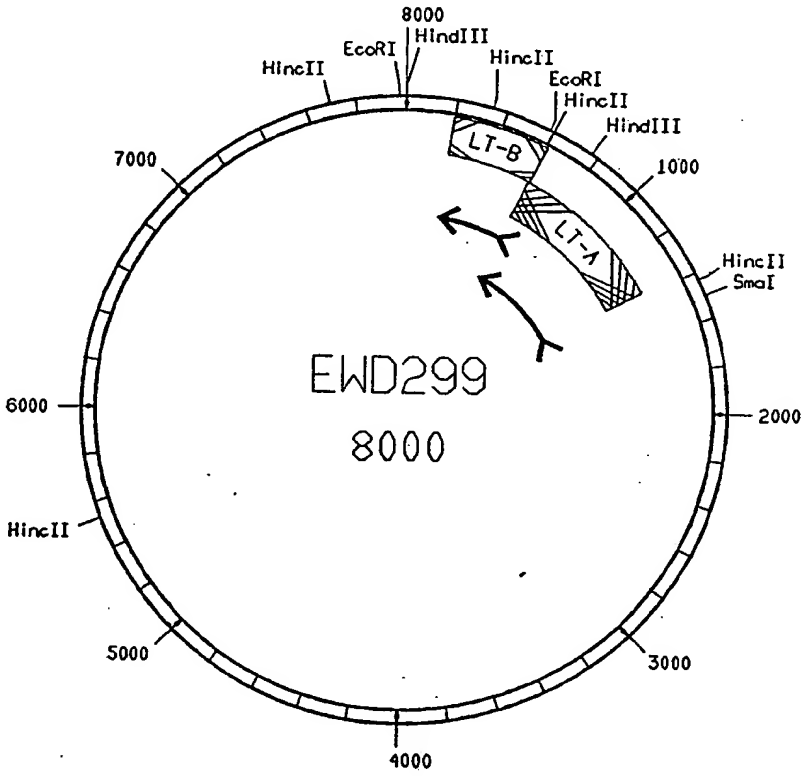
(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IMMUNOGENIC DETOXIFIED MUTANTS OF CHOLERA TOXIN AND OF THE TOXIN LT, THEIR PREPARATION AND THEIR USE FOR THE PREPARATION OF VACCINES

(57) Abstract

An immunogenic detoxified protein comprising the amino acid sequence of subunit A of cholera toxin (CT-A) or subunit A of an *Escherichia coli* heat labile toxin (LT-A) or a fragment thereof wherein one or more amino acids at, or in positions corresponding to Val-53, Ser-63, Val-97, Tyr-104 or Pro-106 are replaced with another amino acid or deleted. Examples of specific replacements include Val-53-Asp, Val-53-Glu, Val-53-Tyr, Ser-63-Lys, Val-97-Lys, Val-97-Tyr, Tyr-104-Lys, Tyr-104-Asp, Tyr-104-Ser, Pro-106-Ser. The immunogenic detoxified protein is useful as vaccine for *Vibrio cholerae* or an enterotoxigenic strain of *Escherichia coli* and is produced by recombinant DNA means by site-directed mutagenesis.



```
LT2      1  -.-.FF-----T----R-A---L---QQ-AYE---PI---   38
LT1      1  ------------------FRS-----.-------------   39
LT1_1A   1  -G-R-------------R--------HN------------   40
CT       1  NDDKLYRADSRPPDEIKQSGGLMPRGQSEYFDRGTQMNIN   40

                --E-----V--NT--N------TVT--Q---I--N--GS-   78
                ---------------------------------Y-----   79
                -------------Y--------L-------A--S----Y   80
                LYDHARGTQTGFVRHDDGYVSTSISLRSAHLVGQTILSGH   80

                NE-----V-P---L-D--G---R---Y-S-N-FA------   118
                -LTIYI---...--------IS------------------   116
                ----------------------V-----Y-----------   120
                STYYIYVIATAPNMFNVNDVLGAYSPHPDEQEVSALGGIP   120

                L---I-----SF-A-EGGMQ---D--GDLF-G-TV--N--   158
                ----------------------------------------   156
                ----------N---I--R-----E------R--N----E-   160
                YSQIYGWYRVHFGVLDEQLHRNRGYRDRYYSNLDIAPAAD   160

                --Q-----SNFP----M--STF--EQ-VPNNKEFK-GV-I   198
                ----------------------------------------   196
                --R------D-Q-------------Q---DSS-TITGD--N   200
                GYGLAGFPPEHRAWREEPWIHHAPPGCGNAPRSSMSNTCD   200

            SA-NV--KYD-MNFKKLL--RLALTFFM--D-F-GVHGE----   241
            -------------------------.-----.-----.----   236
            -E--N-STIY-R------------D-.--EV-.IY---.R---   240
            EKTQSLGVKFLDEYQSKVKRQIFSGY.QSDID.THNRI.KDEL   240
```

Figure 1

Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development

John J. Mekalanos, Daryl J. Swartz & Gregory D. N. Pearson

Department of Microbiology and Molecular Genetics, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115, USA

Nigel Harford, Francoise Groyne & Michel de Wilde

Department of Molecular Genetics, Smith-Kline-R.I.T., rue de l'Institut, 89, B-1330 Rixensart, Belgium

Nucleotide sequence and deletion analysis have been used to identify the regulatory and coding sequences comprising the cholera toxin operon (ctx). *Incorporation of defined* in vitro-*generated* ctx *deletion mutations into* Vibrio cholerae *by* in vivo *genetic recombination produced strains which have practical value in cholera vaccine development.*

MODERN history has recorded seven world pandemics of cholera, a diarrhoeal disease produced by the Gram-negative bacterium *Vibrio cholerae*[1]. Laboratory tests can distinguish two biotypes of *V. cholerae*, classical and El Tor, the latter being responsible for the most recent cholera pandemic. The diarrhoeal syndrome induced by colonization of the human small bowel by either biotype of *V. cholerae* is caused by the action of cholera toxin, a heat-labile enterotoxin secreted by the growing vibrios[2]. Diarrhoeal diseases affecting both humans and animals caused by some enterotoxinogenic strains of *Escherichia coli* are also induced by a heat-labile enterotoxin (LT) which is closely related to cholera toxin in structure and mode of action[1-3].

Cholera toxin is an 84,000-molecular weight (MW) protein composed of one A subunit (27,000 MW) and five B subunits (11,600 MW). The A subunit, although synthesized as a single polypeptide chain, is usually proteolytically nicked to form two disulphide-linked polypeptides, A1 (22,000 MW) and A2 (5,000 MW)[4,5]. The A1 polypeptide is an enzyme and promotes the activation of adenylate cyclase in target cells by catalysing the ADP-ribosylation of a GTP-binding regulatory component of the cyclase complex[6]. The resulting accumulation of cyclic AMP in the intestinal mucosa leads to the severe fluid loss characteristic of cholera. Each B subunit has a high binding affinity for the toxin's cell surface receptor, ganglioside GM_1 (ref. 7). Neutralizing antibodies raised against the holotoxin react mainly with the B subunits[1,2].

Much of the current interest in the genetics of cholera toxin has been promoted by the need to develop a more efficacious vaccine against this enterotoxic disease. Parenterally administered, killed whole-cell and toxoid vaccines have been shown to be largely ineffective in producing long-lasting immunity to cholera, presumably because they lack the ability to induce local immune responses in the intestine[8,9]. Since the natural disease is capable of inducing prolonged immunity[9,10], several investigators have proposed the use of attenuated, non-toxinogenic mutants of *V. cholerae* as live oral cholera vaccines[11-16]. While encouraging results in volunteer studies have been obtained with some of these strains, factors such as genetic instability or poor colonizing ability have contraindicated their use in the field[15-17].

The recent relaxation of US governmental guidelines prohibiting the molecular cloning of bacterial toxin genes has permitted the use of a powerful new approach to the analysis of cholera toxin gene structure and vaccine development. These studies have shown that like the *elt* genes, which encode *E. coli* LT[18], the genes for the A and B subunits of cholera toxin are arranged in a single transcriptional unit with the A cistron (*ctxA*) preceding the B cistron (*ctxB*)[19]. *V. cholerae* strains of the classical biotype contain a nontandem, chromosomal duplication of the *ctx* operon that is structurally identical in all strains.[53] In contrast, about 70% of El Tor strains have only a single copy of *ctx*, while the remaining strains have two or more *ctx* copies present on a tandemly repeated genetic element. This genetic duplication and amplification of the toxin operon may be related to the instability observed in some of the earlier *V. cholerae* toxin mutants[13,16].

In this article, we report the entire nucleotide sequence of one *ctx* operon together with partial sequences containing the *ctx* promoter regions of five other cloned *ctx* copies. Deletion analysis has allowed the identification of toxin transcriptional and translational regulatory sequences. An *in vitro*-constructed, internal deletion in *ctxA* was recombined *in vivo* into both *ctxA* gene copies of *V. cholerae* strain Ogawa 395. Since this genetic recombinant still produces the immunogenic B subunit of the toxin, it should have practical value in cholera vaccine development.

Molecular cloning of *ctx* operon copies

A total of six *ctx* copies were cloned from four *V. cholerae* strains. These include both *ctx* copies from strain 569B, both copies from strain RV79, one of two copies from strain E7946 and the single *ctx* copy of strain 2125. With the exception of the classical strain 569B, all these strains are El Tor in biotype. The *ctx* copies were cloned as various *V. cholerae* restriction fragments which hybridized with ^{32}P-labelled *elt* or *ctx* probes as described in the legend to Fig. 1.

The restriction sites for several endonucleases were located on these cloned inserts, and the resulting maps were aligned at the conserved *Xba*I site previously determined to lie early in the A cistron[19], (Fig. 1*A*). Other conserved restriction sites for *Nru*I, *Pst*I, *Ava*I and *Bgl*II were also found preceding *ctxA* on these various inserts (Fig. 1). Additional restriction mapping and hybridization analysis has indicated that the 5 kilobase pairs (kbp) of DNA directly preceding the toxin structural genes is the same for all cloned *ctx* copies thus far examined[53]. Since the larger chromosomal sequence environment flanking these different *ctx* copies appears to vary as determined by Southern blot hybridization, we have proposed that the conserved DNA immediately upstream of *ctx* is part of a genetic element responsible for toxin operon duplication and transposition events. From a practical point of view, the conserved 5′ flanking sequences associated with *ctx* provided part of the necessary homology for efficient *in vivo* recombination of *in vitro* constructed deletion mutations into multiple copies of the toxin operon (see below).

Nucleotide sequence of *ctx*

The strategy used to determine the complete nucleotide sequence of the single *ctx* copy of strain 2,125 is shown below the restriction map of the insert cloned on pRIT10824 (Fig. 1*B*). The 2,020 nucleotides determined are shown in Fig. 2. Comparison of the 2,125 nucleotide sequence with both the *elt* nucleotide sequence[20,21] and known amino acid sequences of

0028-0836/83/490551—07$01.00

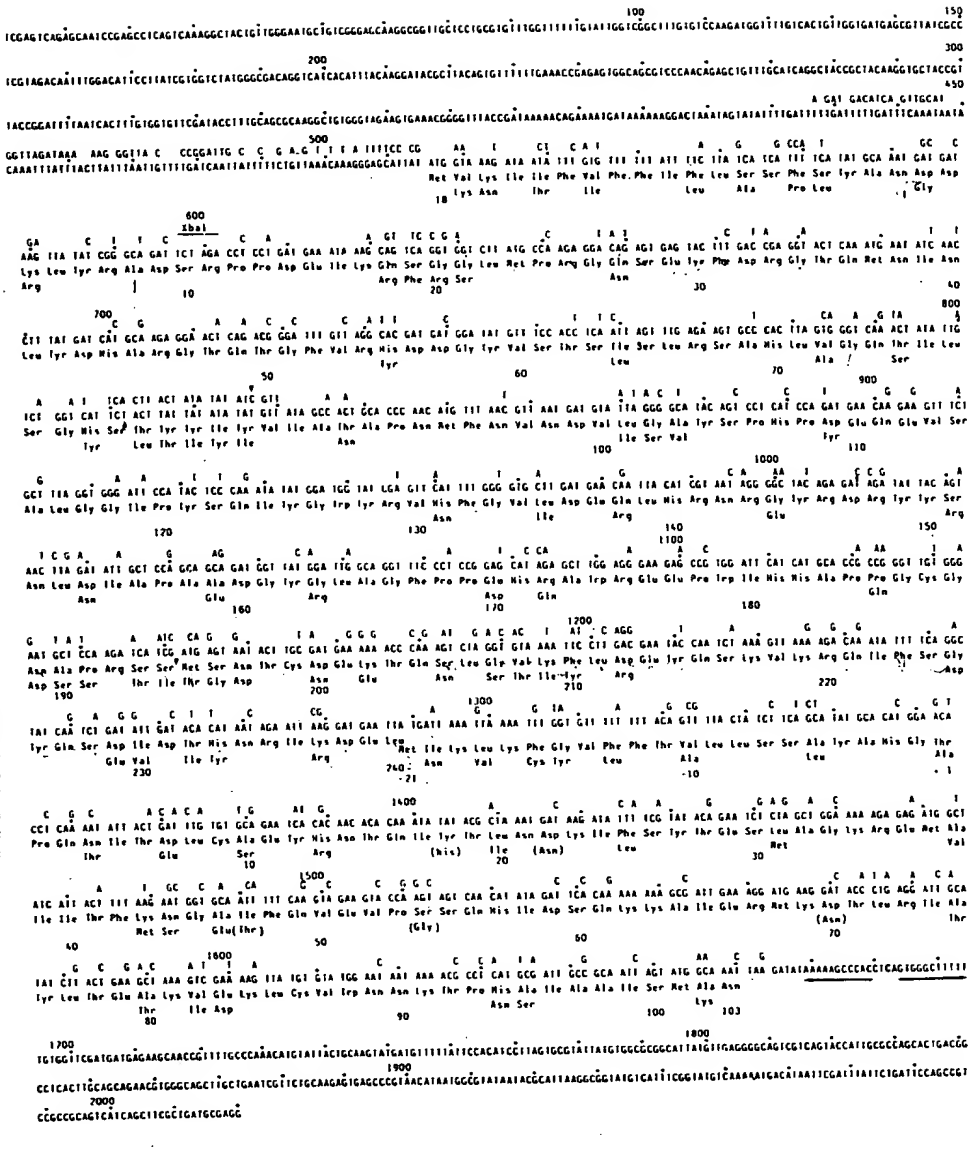


Fig. 2 DNA sequence of the *V. cholerae* toxin operon from strain 2125. The antisense strand is shown from 5′ to 3′. From nucleotide 427 to 1,663, the sequence is compared with the published sequence of LT genes[20,21]; *elt* nucleotides are shown above the sequence only where they differ from the *ctx* sequence except between nucleotides 810 and 830, where deletion of a T (arrowed) in the *elt* sequence creates a frameshift which is corrected by insertion of a C (arrowed) 16 bp downstream (see text). Analogous events have previously been seen after pseudoreversion of frameshift mutations[45] but to our knowledge, this is the first naturally occurring case described. The deduced amino acid sequence of *ctxA* (nucleotide 516 to 1,289) and *ctxB* (nucleotide 1,289 to 1,660) is shown and compared with that of LT. Amino acids that differ in LT are shown below the cholera toxin amino acid sequence. In addition, for the mature B subunit sequence, differences from the published amino acid sequence of B subunit purified from strain 569B[26,27] are shown in brackets. Two of these differences (amino acids 47 and 54) are also found in LT. The cleavage site between the A1 and A2 polypeptides is indicated by an arrow (amino acids 194–195). Note also the overlap of *ctxA* and *ctxB* cistrons (nucleotides 1,289–1,292). Sequence exhibiting dyad symmetry and potentially involved in transcription termination is indicated with divergent arrows. Features of the sequence immediately upstream of the *ctxA* gene are detailed in Fig. 3.

sequence (nucleotides 1,277–1,282, Fig. 2) of the *ctxA* cistron. The first two nucleotides of the *ctxA* translation termination signal TGA are the last two nucleotides of the *ctxB* translation initiation triplet ATG. This particular overlapping arrangement is also found several times in phage λ operons[30] and may be involved in translational coupling[31] of the *ctxA* and *ctxB* genes. However, evidence presented below suggests that this is not the case with the *ctx* operon. Where documented, translational coupling is observed between cistrons whose gene products interact in a one to one stoichiometry[31], and in contrast, the cholera toxin molecule is composed of one A subunit and five B subunits. Moreover, *E. coli* produces stoichiometrically 7 times more cholera toxin B subunit than A subunit (data not shown). Fusion of the *ctxB* gene to various *E. coli* promoters allows high expression of *ctxB* in the absence of *ctxA* translational initiation signals. These data suggest that translation of *ctxB* relies primarily on independent initiations promoted by its own ribosome binding site.

Another experiment supports this conclusion. Our DNA sequencing analysis identified two *Nde*I sites at positions 561 and 1,337 within the *ctxA* and *ctxB* genes, respectively. The positions of these sites relative to the reading frames of *ctxA* and *ctxB* allowed us to construct a *ctxA* deletion which codes for an in-frame fusion of amino acid 17 of the A subunit signal sequence to amino acid 19 of the B signal and thus maintains the normal processing site of the B signal sequence (residue 21). This genetic fusion makes B subunit expression dependent on the efficiency of the A cistron translation initiation sequences, provided the hybrid signal sequence is processed at normal efficiency. *Nde*I digestion of plasmid pGP3 followed by ligation produced such a fusion between these two sites and gave plasmid pJM3.1. Plasmid pJM3.1 produced 0.056 μg ml^{-1} of B subunit in *E. coli* MS371 while pGP3 produced 0.50 μg ml^{-1}. These data suggest that the *ctxB* ribosome binding site is about ninefold more efficient than the *ctxA* site.

Toxin promoter regions

We determined approximately 200 base pairs of sequence upstream of the *Xba*I sites for each of the other five additional cloned copies of the *ctxA* gene, cloned on plasmids pGP3, pGP4, pGP5, pGP6 and JM17. Comparison of these sequences with the corresponding region of the *ctxA* gene derived from strain 2,125 indicated a perfect conservation of sequence between these copies from nucleotides 413 to 590 with one notable exception. The sequence TTTTGAT comprising nucleotides 419–425, 426–432 and 433–439 of the 2,125 sequence was found tandemly repeated 3–8 times preceding different *ctxA* gene copies (Fig. 3). Figure 3 shows part of a sequencing gel autoradiograph that spans DNA carrying eight of these tandem repeats in the region adjacent to the *ctxA* gene of pJM17.

To determine the position of the toxin operon promoter with respect to these repeated sequences, we used nuclease *Bal*31